16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Structure and Interactions of Proteins in Solution	NIH-GM 14603	$236,538 (current year: $37,564) reduced from $44,192; subsequent years equally reduced.	9/71-8/31/76
Thermodynamics and Geometry of Protein Interactions"	NSF-GB 38544X	$ 90,000 (Total cost for 3 years	6/1/73-5/31/76
*The Interaction of Brain Microtubule Protein with Vinblastine and Other Anti-Cancer Drugs	American Cancer Soc. Mass. Division	$ 12,750 This grant is limited to one year and cannot be renewed.	1/1/74-12/1/74

*Largely to support one postdoctoral research associate for one year.

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Tubulin Associations and effects of anti-cancer drugs	NIH- decision pending	$63,526 REQUESTED	11/1/74-10/31/75

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name SERGE N. TIMASHEFF

Signature Serge N. Timasheff Date Jan. 28, 1974

Telephone 617-647-2720
Area Code Number Extension

Checks payable to

Bernard I. Levinson
Coordinator of Sponsored Research

Mailing address for checks

Brandeis University
Waltham, Mass. 02154

Responsible officer of institution

Typed Name Marver H. Bernstein

Title President

Signature Marver H. Bernstein Date 1/24/74

Telephone 617-647-2201
Area Code Number Extension

10035403G1

Source: https://www.industrydocuments.ucsf.edu/docs/pjll0000

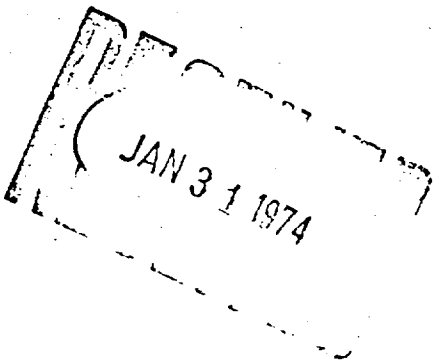
ack
1/31/74
gk

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date:
January 25, 1974



1. Principal Investigator (give title and degrees):

Professor Serge N. Timasheff, Ph.D.

2. Institution & address:

Brandeis University
Waltham, Massachusetts 02154

3. Department(s) where research will be done or collaboration provided:

Graduate Department of Biochemistry

4. Short title of study:

Interactions of microtubules with Nicotine and Nicotine Products

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

It is proposed to undertake a detailed study of the mechanism on the molecular level of binding of nicotine and products of nicotine metabolism to tubulin, the subunit protein of microtubules, and of the effects of this complexation on the self-assembly of the microtubule protein subunits which leads to microtubule formation. Specifically, the interactions of tubulin with nicotine, its liver metabolites and chemical derivatives will be examined quantitatively by column chromatographic and spectroscopic techniques. The self-association of tubulin in the presence of these ligands will be examined by sedimentation, light scattering, calorimetric and column chromatographic techniques. The results will be analyzed in terms of most modern rigorous thermodynamic theory. Furthermore, the effect of these tobacco products on the morphology of microtubules will be studied by electron microscopy, and their interference with the self-assembly of these organelles will be followed by in vitro microtubule reconstitution experiments. Such knowledge will advance the understanding of the mechanism by which nicotine affects biological processes. It is only with such an understanding that a rational approach can be made eventually to the question of the prevention of harmful action.

1003540352

Source: https://www.industrydocuments.ucsf.edu/docs/pjfl0000

gations will be performed by sedimentation velocity. The dependence of the sedimentation coefficient and of the area distribution under the reaction boundary on both protein and nicotine concentrations will be determined. The results of this study will be analyzed in terms of the theory of sedimentation for ligand-mediated association of Cann and Goad (161) and the simulation of sedimentation patterns of associating systems, as described, for example, by Gilbert and Gilbert (155). The molecular weight distribution as a function of protein concentration will be obtained from sedimentation equilibrium studies. This will enable the calculation of the equilibrium constant of this system. Measurements as a function of temperature will yield the van't Hoff enthalpy, and, therefore, the entropy of the system. Furthermore, microcalorimetric studies of this association process, as well as of dilution-induced dissociation of the aggregates (44), will provide additional valuable thermodynamic information for furthering our understanding of these processes.

Following the indications of Table 1, the effects of ionic strength, specific anions, D_2O and non-polar solvents on the protein interactions in the presence of nicotine will also be investigated. The interpretation of such measurements may require the application of multicomponent theory to include the consideration of preferential interaction of solvent components with the protein (62,63).

Following the studies with nicotine itself, similar measurements will be carried out with nicotine derivatives, following as a guide the results of the binding studies described above.

As knowledge develops on the tubulin-nicotine interaction, the solution studies will be extended to the level of self-assembly of microtubules in vitro. Reconstitution experiments will be carried out according to the method of Weisenberg (64). The system will contain in addition nicotine or a related compound identified through solution studies as a perturber of tubulin self-association. It is expected that such perturbation on the molecular level should have a strong effect on the reconstitution of microtubules and on the morphology of assembled structures, if such are formed in the presence of the ligands.

The results of these experiments should permit to carry out well controlled in vitro experiments on the effects of self-assembly perturbing nicotine-related ligands on reconstituted microtubules. In these experiments, microtubules will be reconstituted according to the method of Weisenberg (64), ligand will be added to the system and a kinetic study of microtubule degeneration will be carried out by changes in light scattering and in details of structures observed by electron microscopy. In the late stages of microtubule degeneration, structures sufficiently small to be followed by solution

1003540358

Source: https://www.industrydocuments.ucsf.edu/docs/pjk0068

thermodynamic and conformational methods could be formed. This would complete the cycle of experiments. In this manner, it is expected that a direct link should be established between the quantitative solution studies on the molecular level and the morphological effects.

Effects of nicotine and nicotine-related compounds on the structural stability of tubulin.

Since the binding of ligands to a protein may induce conformational changes, the thermodynamic studies will be complemented by careful conformational examination of the various systems. These experiments will be done using the methods of circular dichroism and fluorescence and, if necessary, NMR. Preliminary experiments in the principal investigator's laboratory indicate that, in the near ultraviolet region, some rotational bands are perturbed in the circular dichroism spectrum by the binding of the alkaloid vinblastine. Similar perturbations may be expected to accompany the binding of nicotine. While the affected bands are most likely due to tyrosine transitions, their identification will be carried out by controlled chemical modification of the tyrosines by acetylatian and cyanuration (65), followed by binding experiments and circular dichroism and fluorescence measurements.

Furthermore the effect of nicotine binding on the structural stability of tubulin will be examined by parallel thermodynamic, conformational and activity experiments as a function of ligand composition. The thermodynamic experiments will be aimed at the determination of the transition temperature of tubulin. The transition temperature will be determined by monitoring the change in UV absorption in the 280 nm region and treating the results by standard thermodynamic methods. Densimetry measurements will also be carried out. These will result in a measurement of the volume change, ΔV, which occurs when nicotine is bound to tubulin, since (66)

$$\Delta V = M_2 \left[\bar{v}_2 \text{ (in the presence of nicotine)} - \bar{v}_2 \text{ (in water)}\right]$$

where M_2 is the molecular weight of the protein.

The thermodynamic information available from the transition temperature measurements, supplemented by microcalorimetric measurements, on the effects of nicotine on the stability of the tubulin molecule, together with the knowledge of the energy of stabilization of the system by the ligands and of the change in apparent volume of the protein obtained from the densimetry measurements, should lead to a further understanding of the nature of the interactions and of the manner in which nicotine and related compounds affect the stability of the tubulin molecule.

10035540359

Source: https://www.industrydocuments.ucsf.edu/docs/pjl0000

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Dr. Serge N. Timasheff, Prof. and Principal Investigator	15	-
Dr. Marina Gorbunoff, Senior Research Associate	20	$ 3,600
Dr. James Lee, Sr. Research Asso.	20	-
Dr. Donald Atha, Research Asso.	100	9,500
To be appointed: Grad. Research Fellow	100	3,500
Technical		
Valda Bolis, Research Technician	100	8,000
Miriam Torney, Adm. Assistant	20	1,500
Staff: Dishwasher	20	1,250
Fringe Benefits		2,188
	Sub-Total for A	29,538

B. Consumable supplies (by major categories)

Glassware	1,000
Chemicals and Biochemicals	2,000
Chromatographic Supplies	1,500
Replacement cells, Xenon lamps and liquid nitrogen for Cary 60 Spectropolarimeter	1,500
Sub-Total for B	6,000

okay gk

C. Other expenses (itemize)

Travel to pertinent meetings	1,000
Publication costs	1,000
Servicing of equipment	900
Xeroxing and drafting costs	500
Sub-Total for C	3,400
Running Total of A + B + C	38,938

D. Permanent equipment (itemize)

LKB Fraction collector UVcord column monitoring device, recorder and other accessories	7,000
Replacement drives and rotors for analytical ultracentrifuge	1,000
Sub-Total for D	8,000
E	5,512
Total request	52,450

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	31,015	7,000	3,600	1,000	6,242	48,857
Year 3	32,536	7,500	3,800	1,000	6,575	51,411

10035403 60

Source: https://www.industrydocuments.ucsf.edu/docs/pjll0000

the various types of forces which contribute to the binding.

The interactions between biological molecules involve many complex reactions of covalent and noncovalent forces, such as dipole-dipole interactions, hydrogen bonds, hydrophobic and Coulombic interactions. These may be identified and sorted out by subjecting the associating system to various probes and monitoring the response of the system. The thermodynamic characteristics of the various types of non-covalent interactions and the probes that can be used to distinguish between them are summarized in Table I (39,41).

TABLE I

Intermolecular Forces

1. Electrostatic			
a. Non-specific	ΔG^o	$>o$	Vary pH and ionic strength
b. Specific site	ΔG^o	$<o$	Vary pH and ionic strength
	ΔH^o	$<o$	Chemical modification of site
	ΔS^o	$>o$	Titration of specific groups
			Vary nature of supporting electrolyte ($F^- < Cl^- < ClO_4^-$)
2. H-Bonding	ΔG^o	$<o$	Vary temperature
	ΔH^o	$<o$	Identify groups by chemical
	ΔS^o	$<o$	and spectroscopic means
3. Hydrophobic	ΔG^o	$<o$	Vary temperature
	ΔH^o	$>o$	Vary medium (D_2O,
	ΔS^o	$>o$	Hofmeister series:
	ΔC_p	$<o$	$SCN^- < Cl^- < SO_4^=$)
4. van der Waals	Small		

In probing intermolecular interactions, a particularly valuable approach is through variations of the medium. By altering the polarity of the medium, such as by addition of organic solvents, the dielectric constant of the medium would be decreased. This strengthens the electrostatic interactions, but weakens the hydrophobic interactions. Another sensitive probe is available in the Hofmeister series of anions (142). In general, the anions may be listed as a series in increasing degrees of strengthening hydrophobic interactions: SCN^-, $ClO_4^- < Br^- < Cl^-$, $AC^- F^- < HPO_4^=$, $SO_4^=$. It can be expected, however, that their effect on ion pair formation should be just the opposite (143).

10035403356

Source: https://www.industrydocuments.ucsf.edu/docs/pjll0000

The pH-dependence of the binding will be followed. This will give information on ionizable groups involved in the process. Changes in the state of ionization of microtubule protein during interactions can also be followed calorimetrically. In buffered aqueous solutions, the observed calorimetric heat, Q_{obs}, is given by the relation

$$Q_{obs} = Q_{rx} + n_H Q_{ion}$$

where Q_{rx} is the heat of the reaction, Q_{ion} is the heat of ionization of the buffer, and n_H is the number of protons involved. If a reaction is studied at constant pH in buffers of different heats of ionization, then n_H AND Q_{rx} may be readily determined. The sign associated with n_H indicates whether protons are released or absorbed during the reaction. This powerful method has been used in a number of studies (44,46) and has revealed the presence of reactive protons not previously suspected.

Following the studies with nicotine itself, similar measurements will be carried out with pyridine and N-methylpyrrolidine, as well as with chemical derivatives of nicotine, with chemical modifications dictated by the conclusions of the thermodynamic studies.

Effects of nicotine and nicotine-related compounds on the self-association properties of tubulin.

The tubulin-nicotine binding studies will be paralleled by studies of the perturbation of the self-association of tubulin to form microtubules. Since normal intact microtubules are required for their physiological function, a perturbation of the morphology of these structures or in the pathways of their assembly results in serious perturbations of biological function.

Studies in the principal investigator's laboratory indicate that, in the presence of magnesium ions, tubulin self-associates in steps to a species that, in size, is similar to the discs that have been reported as precursors of microtubule formation. Therefore, the effect of nicotine binding on tubulin self-association in the presence of magnesium will be investigated, in a manner similar to the studies in the principal investigator's laboratory of the perturbation of magnesium-induced association of tubulin by the alkaloid, vinblastine (23).

The association studies will be pursued primarily by sedimentation (47-55) and light scattering (56-60), since the data obtained by any one technique alone may not be sufficient to elucidate clearly the behavior of the system. The initial investi-

1003540357

Source: https://www.industrydocuments.ucsf.edu/docs/pjll0000

8. Brief statement of working hypothesis:

While nicotine is known to have strong and complex pharmacological effects both on the central and the peripheral autonomic nervous systems, its mechanism of action at the molecular level is not understood (1,2). Recently, it has been found in our laboratory that nicotine binds strongly to tubulin, the protein subunit of microtubules (3). Microtubules have been identified in recent years as key components of most cells (4,5,6). They are found in the axons, dendrites and neurites of nerve cells (7,8,9) and have been implicated in sensory transduction (10); they form part of the mitotic spindles (11,12,13) of dividing cells, and they are involved in maintenance of cell shape and in the intracellular transport of material (14,15). Microtubules are not static entities, but they are in dynamic equilibrium with subunits in the cytoplasm (16,17,18,19), being assembled as required by cell function. The structure of microtubules is known to be perturbed or destroyed by complexation with ligands, such as various plant alkaloids (20,21,22), with the concomitant destruction of physiological function. In view of these facts, it seems of importance to pursue in depth our observation of the binding of nicotine to microtubule subunits and to establish the detailed mechanism of this binding and the nature of related effects on the structure of microtubules and their ability to self-assemble. The gaining of such understanding should significantly advance the elucidation of the molecular mechanism by which nicotine interacts wtih tissues to produce its various pharmocological effects.

9. Details of experimental design and procedures (append extra pages as necessary)

9. Details of experimental design and procedures

The proposed experimental work will consist of the following studies:

1. Binding of nicotine, nicotine metabolites and derivatives of nicotine to tubulin;
2. Effects of nicotine and nicotine-related compounds on the self-association properties of tubulin;
3. Effects of nicotine and nicotine-related compounds on the structural stability of tubulin.

The investigation will be done using calf brain microtubule protein. This is an excellent system for study, since fresh calf brains are readily available, and the isolation procedure for obtaining homogeneous protein is well established (23,24). The purified calf brain microtubule protein will be stored in 1M sucrose, according to the method developed in this laboratory by Frigon and Lee (25) who have demonstrated that this medium stabilizes this protein, which normally is highly unstable and readily denatured. A further advantage of using calf brain microtubule protein is that it has been recently characterized in great detail by Lee et al. (24,26).of this laboratory.

In view of these ground-breaking studies on the protein, this laboratory is at present in a unique position for carrying out the proposed studies.

Lee et al (24) have shown that the chemical composition (amino acid sequence) of calf brain microtubule protein is similar to that of microtubule subunits from other sources. The protein consists of two almost identical subunits, each with a molecular weight of 54,000 ± 1,000. There is one intrachain disulfide bridge and several free

10035403S3

Source: https://www.industrydocuments.ucsf.edu/docs/pjll0000

by similar binding studies of products of nicotine metabolism and other nicotine-related compounds. Initially, the binding of nicotine metabolites to tubulin will concentrate on cotinine and cotinine-N-oxide, since their incorporation in even minute amounts into microtubules will be detectable by radioimmunoassay techniques developed for these compounds, as well as for nicotine, by Dr. H. Van Vunakis of this Department (34,35). In later stages of the investigation, similar studies will be extended to other metabolites. The compounds in question are either available commercially or can be synthesized according to methods described in the literature (35).

In order to establish the mechanism of binding of a ligand to a protein it is essential to determine the stoichiometry of the association and the variation of the equilibrium constant with changes in conditions (e.g. temperature, pH of the medium, nature and concentration of salt in the buffer system, etc.) (36). From the thermodynamic parameters obtained in this way, namely the free energy (ΔG), the enthalpy (ΔH), the entropy (ΔS) and the heat capacity (ΔC_p), and from the patterns of their variations with changes in environment, the nature of the forces operative, and thus of the types of interactions and of the chemical groups involved, can be deduced. These conclusions can be confirmed and further refined by proper chemical modifications of the protein and the ligand followed by binding measurements. For example, groups identified on the protein by thermodynamic measurements can be selectively blocked or altered; on the other hand, groups on the ligand can be modified by the synthesis of the proper derivatives, and the component parts of a complex ligand, such as nicotine, can be studied separately (in the case of nicotine this would involve separate binding studies of pyridine and of N-methylpyrrolidine); such experiments would determine the contribution of each ring of nicotine to the total binding.

The binding studies will be performed using a variety of techniques, selected according to the properties of the individual ligands and to the particular piece of information sought in a given experiment. These will include equilibrium dialysis, ultrafiltration, absorption spectrophotometry, fluorescence and various column methods (e.g., the one described by Hummel and Dreyer (37), and the column scanner developed by Ackers (38)). By applying these experimental methods to a given protein-ligand system, the number of binding sites for the ligand on the protein and the intrinsic binding constant can be determined. By making experimental measurements at several different temperatures, the van't Hoff enthalpy and also the entropy of binding will be calculated. In addition to the equilibrium studies, direct calorimeteric determinations of reaction heats will be performed.

As stated above, in order to arrive at an exact understanding of the mechanism of interaction, it is necessary to sort out

1003540355

Source: https://www.industrydocuments.ucsf.edu/docs/pjl0000

sulfhydryl groups fully accessible to solvent. The native isolated protein consists of dimers with a molecular weight of 110,000. Weisenberg and Timasheff have shown that, in the presence of magnesium, these dimers self-associate to large species (23) with molecular weights in the vicinity of 2.5 million, as found by Frigon and Timasheff (27). This species is similar in size to the disk-like structures which have been observed (28) and proposed as centers of nucleation for the self-assembly of microtubules.

The assembled microtubules are hollow, usually straight cylinders, approximately 240A in diameter. The outer walls are 50A thick and are composed of the tubulin subunits. The assembled microtubules, in vivo, are in a state of dynamic equilibrium with a soluble pool of subunits (18,29). Thus, it appears that these structures are assembled as needed by cellular function.

Binding of ligands to microtubules strongly perturbs or destroys their structure, with a resulting loss of their physiological function. This effect has been studied in particular detail for the plant alkaloids colchicine and vinblastine, which not only destroy these structures in vivo (20,22), but also strongly affect the self-association of tubulin in in vitro solution studies, as shown by Weisenberg and Timasheff (23).

The outlined studies will be carried out by a variety of protein physico-chemical and chemical techniques. These include equilibrium and velocity sedimentation, light scattering, gel filtration, circular dichroism, fluorescence, ultra-violet and infra-red spectroscopy, microcalorimetry, densimetry, controlled chemical modification of protein side chains, potentiometric titration, in their various ramifications as required for each particular study, and with the rigorous quantitative application of the most modern theories in the analysis of the results. These methods, which have been used with a great deal of success in the past in the principal investigator's laboratory, are described well in the review literature, for example in three volumes recently edited by the principal investigator (30) (Tables of contents enclosed.). These techniques do not need to be described in detail here. Suffice it to remark that the criterion used in the past by the principal investigator will be applied to these studies, as well; namely that results obtained on a problem by several unrelated techniques should be quantitatively consistent.

10035403S4

Binding of Nicotine and Nicotine-Related Compounds to Tubulin

Nicotine is actively metabolized by man and other mammals by a variety of pathways to give a number of products (31,32). The principal pathways and products are summarized in Figure 1. It is not clear at present, however, what role the products of nicotine metabolism play in the physiological responses to nicotine (32,33).

Since it has been observed in our laboratory that nicotine binds strongly to tubulin, studies of the mechanism of the binding of nicotine to tubulin will be pursued in detail. These will be supplemented

Source: https://www.industrydocuments.ucsf.edu/docs/pjl0000